Available online at www.sciencedirect.com
ScienceDirect
Behavioural Brain Research 175 (2006) 149–156
BEHAVIOURAL BRAIN RESEARCH
www.elsevier.com/locate/bbr

Research report

Gender specific effects of ethanol in mice, lacking CCK_2 receptors

Urho Abramov [a,b,*], Sirli Raud [a,b], Jürgen Innos [a,b], Sulev Kõks [a,b], Toshimitsu Matsui [c], Eero Vasar [a,b]

[a] *Department of Physiology, University of Tartu, 19 Ravila Street, 50411 Tartu, Estonia*
[b] *Center of Molecular and Clinical Medicine, University of Tartu, 19 Ravila Street, 50411 Tartu, Estonia*
[c] *Division of Hematology/Oncology, Department of Medicine, Kobe University School of Medicine, 650-0017 Kobe, Japan*

Received 9 June 2006; received in revised form 11 August 2006; accepted 15 August 2006
Available online 12 September 2006

Abstract

Neuropeptide cholecystokinin (CCK) has been reported to suppress ethanol intake, but there is contradictory evidence about the role of CCK_2 receptors. In the present study anxiolytic, hypolocomotor and sedative effects of acute ethanol administration, but also voluntary ethanol consumption were studied in male and female mice, lacking CCK_2 receptors (−/−). Ethanol (1.0 and 2.0 g/kg) induced a significant reduction of anxiety-related behaviours in the elevated plus-maze, but this effect was statistically significant only in female homozygous mice (−/−). In male mice, lacking CCK_2 receptors (−/−), but not in their wild-type littermates (+/+), the suppression of vertical locomotor activity was caused by ethanol at a dose 0.5 g/kg. The highest dose of ethanol (2.0 g/kg) produced statistically significant reduction of horizontal locomotor activity only in female wild-type (+/+) mice, but this effect was related to increased basal activity when compared to female mutant (−/−) mice. Duration of the loss of righting reflex was not significantly affected by genotype or gender, but blood ethanol levels at regain of righting reflex were significantly lower in female homozygous mice (−/−) compared to their wild-type (+/+) littermates, indicating increased sensitivity to the sedative effect of ethanol. Ethanol intake, but not preference, at concentration 10% was significantly increased in female mice, lacking CCK_2 receptors (−/−). The present study revealed an altered response to the acute effects of ethanol in CCK_2 receptor deficient mice (−/−). These changes are gender-specific and could be attributed to the altered activity of dopaminergic system in male mice and increased activity of GABA-ergic system in female mice as established in our previous studies.

Keywords: Ethanol; CCK_2 receptor-deficient mice; Anxiety; Locomotor activity; Sensitivity; Ethanol intake

1. Introduction

Cholecystokinin (CCK) is a gastrointestinal peptide widely distributed in the central nervous system [35]. CCK is involved in the regulation of various physiological functions in the brain, such as modulation of anxiety and stress-related behaviours, regulation of feeding, nociception, memory and reward-related behaviours [7,13,36]. There is substantial evidence that CCK acts as a neurotransmitter and that it exerts a modulatory influence through several classic neurotransmitters including dopamine, GABA and opioid peptides [6]. By now two subtypes of CCK receptors have been identified. CCK_1 receptors are located in the pancreas, gallbladder and distinct brain regions. CCK_2 receptors represent the vast majority of CCK receptors found in the central nervous system [30].

CCK has been reported to suppress ethanol drinking and preference in rodents [15,16,20,39] and the role of CCK_1 receptor has been underlined [8,9,27]. Studies concerning the role of CCK_2 receptors in ethanol preference have yielded contradictory results. Crespi [8] described that pre-treatment with CCK_2 antagonists did not affect ethanol drinking in rats. However, Little et al. [23], and Croft et al. [11] reported that CCK_2 antagonists decreased stress-induced ethanol preference in mice. Ethanol consumption has recently been studied in male mice lacking CCK_2 receptors, but no differences compared to wild-type (+/+) mice have been established [27]. On the other hand, chronic ethanol consumption has been shown to alter the brain CCK-ergic system [18,41] and CCK_2 receptors have been reported to have relevance in ethanol withdrawal-induced anxiety and convulsions [5,42,43].

* Corresponding author at: Department of Physiology, University of Tartu, 19 Ravila Street, 50411 Tartu, Estonia. Tel.: +372 7 374 335; fax: +372 7 374 332.
E-mail address: urhoa@ut.ee (U. Abramov).

0166-4328/$ – see front matter © 2006 Elsevier B.V. All rights reserved.
doi:10.1016/j.bbr.2006.08.015

In the present study, female and male homozygous (−/−) CCK_2 receptor deficient mice were used to reveal a possible role of CCK in ethanol-related behaviours. Previous studies have established that male CCK_2 receptor deficient mice display an increased sensitivity of dopamine D_2 receptors in the striatum and an altered response to dopaminergic drugs such as amphetamine and apomorphine [12,19]. Altered responses to opioid- and GABA-ergic drugs along with distinct changes in opioid- and GABA-ergic systems have also been reported [12,32–34,38,40]. Considering that as a result of significant neurochemical alterations mice lacking CCK_2 receptors display altered responses to such commonly abused drugs as amphetamine, morphine and diazepam [12,19,34], it was assumed that ethanol-related behaviours together with ethanol consumption would also be modified due to the changes in opioid-, dopamine- or GABA-ergic systems. The study was carried out employing both male and female mice, because the phenotype of mice, lacking CCK_2 receptors, is significantly affected by gender [1]. Behavioural effects of ethanol were studied in the elevated plus-maze, locomotor activity and loss of righting reflex tests. Also, ethanol consumption and preference, and preference for non-alcoholic tastants were determined.

2. Materials and methods

2.1. Animals

CCK_2 receptor deficient mice were provided from the original background 129sv/C57BL6 mice [26]. CCK_2 receptor deficient mice were generated by homologous recombination by replacing a part of exon 2 and exons 3–5 [29]. Breeding and genotype analysis were performed in the Department of Physiology, University of Tartu [19]. Male and female homozygous (−/−) CCK_2 receptor-deficient and wild-type (+/+) mice (90 days old) were used in the behavioural experiments. Mutant mice were crossed back six times to the C57/BL6 background to minimise the possible genetic effects from the 129Sv strain. Mice were kept in the animal house at 20 ± 2°C under a 12:12 h light/dark cycle (lights off at 19:00 h). Tap water and food pellets were available *ad libitum*. In all experiments ethanol-naïve mice were used. All animal procedures were approved by the University of Tartu Animal Care Committee in accordance with the European Communities Directive of 24 November 1986 (85/609/EEC).

2.2. Elevated plus-maze

The number of animals per group was $N = 8$–10. The mouse elevated plus-maze is a reduced copy of the rat plus-maze [17]. The plus-maze consists of two opposite open (17.5 cm × 5 cm) arms without sidewalls and two enclosed arms of the same size with 14 cm high sidewalls and an end wall. To determine the exploratory activity, the open arms were divided into three equal parts by lines. The entire plus-maze apparatus was elevated to a height of 30 cm and placed in a brightly lit room (illumination level: ∼500 lx in open arms). Standard 5 min test duration was employed [22], and the maze was wiped with damp and dry towels between the subjects. Test sessions were video-recorded and the videotapes were subsequently scored by a trained observer unaware of testing conditions. The following parameters were observed: (1) number of entries on the open arms; (2) time spent on the open arms of the plus-maze; (3) total number of closed and open arm entries; (4) ratio between the open and total arm entries; (5) number of unprotected head-dips; (6) number of lines crossed. Time spent on the open arms, number of open arm entries, and ratio between the open and total arm entries are the conventional measures of anxiety in the elevated plus-maze [22]. Three doses of ethanol (0.5, 1.0 and 2.0 g/kg) were studied. Ethanol (diluted in physiological saline, 10% (v/v) for 0.5 and 1.0 g/kg or 20% (v/v) for 2.0 g/kg) was injected intraperitoneally 20 min prior to testing.

2.3. Locomotor activity

The number of mice was $N = 10$–12 per treatment group. For the study of locomotor activity the animals were placed singly into soundproof photoelectric motility boxes (448 mm × 448 mm × 450 mm) connected to a computer (TSE; Technical & Scientific Equipment GmbH, Germany) for 30 min. The illumination level of the transparent test boxes was ∼400 lx. After removing the mouse from the box, the floor was cleaned with damp towels and dried thoroughly. Time in locomotion (s), distance travelled (m) and number of rearing were registered. Three doses of ethanol (0.5, 1.0 and 2.0 g/kg) were used. Ethanol was injected intraperitoneally 20 min prior to testing.

2.4. Loss of righting reflex

Mice ($N = 14$–15 per group) were given an intraperitoneal injection of 4.0 g/kg of ethanol (20%, v/v). At the onset of ethanol-induced sedation (the loss of righting reflex), each mouse was placed on its back in a V-shaped paper-trough. Time (s) between the injection and the loss of righting reflex and time (min) between the loss of righting reflex and the regain of righting reflex defined as the ability to right itself on all four paws three times within a 30 s interval were taken. Tail blood samples were collected at the regain of righting reflex to determine blood ethanol concentration.

2.5. Ethanol intake test

Fifty millilitres plastic tubes with tips cut off were used for ethanol intake and taste preference tests. Tubes were controlled for leakage for 7 days and subsequent intake measurements were adjusted for leakage. Throughout the experiment, total fluid and food intake, and body weight were measured every 7 days. The number of mice was $N = 15$ per group. Prior to testing mice were housed individually and were habituated to drinking from two tubes containing plain water for 7 days. Mice were then given 24 h access to two tubes, one containing plain water and the other containing ethanol in water. The concentration of ethanol (v/v) was increased every 7 days. Initially, mice received 3%, followed by 6% and finally 10% ethanol solution. The positions of the tubes were counterbalanced between groups and changed every 2 days to control for position preference. Average ethanol consumption (calculated in g/kg of body weight per day) was obtained for each ethanol concentration by weighing tubes at the beginning and end of the exposure. As a measure of relative ethanol preference, an ethanol preference ratio was calculated for each ethanol concentration by dividing total ethanol solution consumed by total fluid (ethanol plus water) consumption. Food intake was calculated weekly at every ethanol concentration (expressed as g/kg of body weight per day) by weighing food granules at the beginning and end of the exposure to ethanol. All spillage was collected and included in calculations.

2.6. Taste preference

Ten days after the end of ethanol consumption testing, the same mice used in alcohol intake test were given *ad libitum* access to two tubes, one containing plain water and the other a solution of sucrose or quinine. The compounds were presented in the following order: sucrose solutions (1.70% and 4.3%) followed by quinine solutions (0.03 and 0.10 mM). Mice had 48 h access to each solution, the position of solutions was counterbalanced between groups and switched 24 h after presentation. Millilitres of solution consumed per kilogram of body weight per day and preference for either compound were measured and calculated as described in the previous section.

2.7. Blood ethanol concentrations

Five microlitres of blood was taken from the tail vein 30, 60, 120 and 240 min after intraperitoneal injection of ethanol (2.0 or 4.0 g/kg 20% (v/v), diluted in physiological saline). Each group consisted of six animals. Blood samples were analysed immediately by enzymatic colour test using LKM 139 and miniphotometer LP 20 (Dr. Bruno Lange GmbH, Germany) according to manufacturer's instructions.

2.8. Statistical analysis

Results are expressed as mean values ± S.E.M. The results of the elevated plus-maze and locomotor activity test were analysed using three-way independent-groups ANOVA (genotype × gender × ethanol treatment). The loss of righting reflex and blood ethanol concentration at regain of righting reflex were analysed by means of two-way independent-groups ANOVA (genotype × gender). Three-way mixed-design ANOVA (genotype × gender × concentration) with two between-subjects variables (genotype and gender) and one within-subjects variable (concentration) was used to analyse the results of the ethanol intake test, and preference for sucrose and quinine. Finally, four-way mixed-design ANOVA (genotype × gender × ethanol dose × time) with three between-subjects variables (genotype, gender and ethanol dose) and one within-subjects variable (time) was applied to analyse blood ethanol concentrations. *Post hoc* comparisons between individual groups were performed by means of Tukey's honest significant difference (HSD) test for either equal or unequal sample sizes using the Statistica for Windows software.

3. Results

3.1. Plus-maze test

(1) *Number of entries on open arms*. Significant effects of ethanol treatment ($F(3, 129) = 9.12$, $p < 0.001$) and gender ($F(1, 129) = 5.94$, $p < 0.05$) were established by application of three-way independent-groups ANOVA (genotype × gender × ethanol treatment). *Post hoc* comparison did not indicate significant differences between groups. However, there was a tendency toward increase in the number of open entries induced by ethanol doses 1.0 g/kg ($p = 0.09$) and 2.0 g/kg ($p = 0.09$) in female mice, lacking CCK_2 receptors (−/−) (Fig. 1A).

(2) *Time spent exploring open arms*. Significant effect of ethanol treatment ($F(3, 129) = 8.03$, $p < 0.001$) and gender ($F(1, 129) = 9.57$, $p < 0.01$) were revealed. The highest dose of ethanol (2.0 g/kg) induced a significant ($p < 0.01$) increase in time spent on open arms in female mice, lacking CCK_2 receptors (−/−), but not in their wild-type (+/+) littermates (Fig. 1B). This effect was not observed in male mice, independent of genotype, either.

(3) *Number of total arm entries*. Three-way ANOVA indicated significant effect of gender ($F(1, 129) = 5.48$, $p < 0.05$), but not of ethanol treatment. Ethanol did not alter the number of total arm entries in any group studied (data not shown).

(4) *Ratio between open and total arm entries*. Significant effects of ethanol treatment ($F(3, 129) = 10.92$, $p < 0.001$) and gender ($F(1, 129) = 4.50$, $p < 0.05$) were observed. However, no significant differences between groups were established by *post hoc* comparison of means (data not shown).

(5) *Number of unprotected head-dips*. The application of three-way ANOVA indicated significant effects of ethanol treatment ($F(3, 129) = 10.91$, $p < 0.001$) and gender ($F(1, 129) = 8.81$, $p < 0.01$), but also a tendency toward the effect of gender × ethanol treatment ($F(3, 129) = 2.62$, $p = 0.053$). Ethanol significantly increased the number of unprotected head-dips in female mutant mice (−/−) at doses 1.0 g/kg ($p < 0.01$) and 2.0 g/kg ($p < 0.01$) (Fig. 1C). This effect of ethanol was not observed in any other group studied.

(6) *Number of lines crossed*. Significant effects of ethanol treatment ($F(3, 129) = 11.21$, $p < 0.001$) and gender ($F(1, 129) = 7.10$, $p < 0.01$) were established. Again, ethanol 2.0 g/kg increased the number of lines crossed significantly ($p < 0.05$) only in female mice, lacking CCK_2 receptors (−/−), but not in their wild-type (+/+) littermates or male mice of either genotype (Fig. 1D).

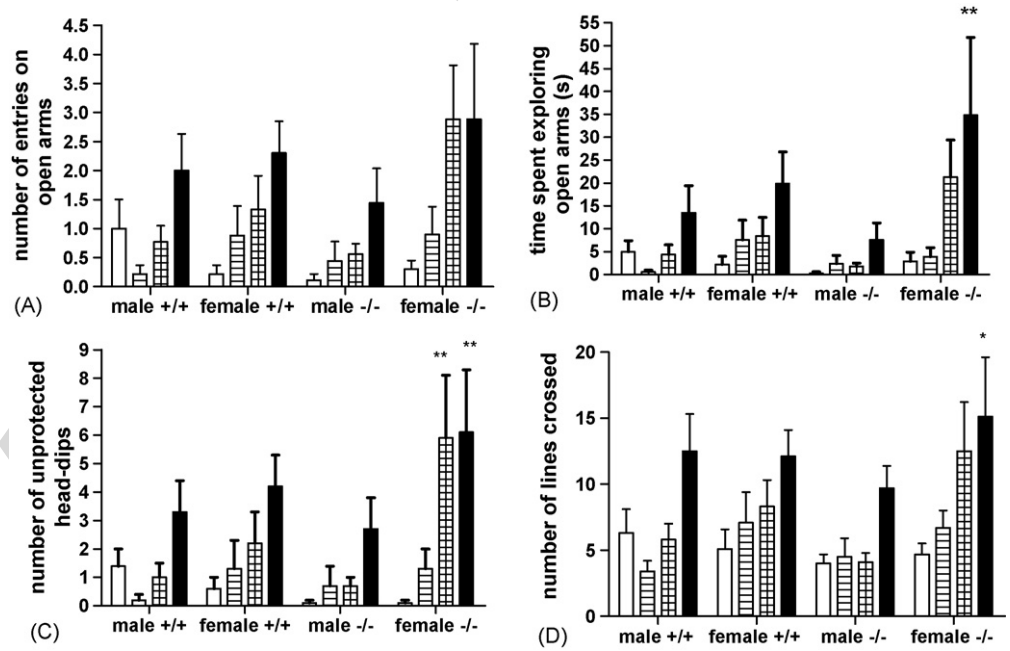

Fig. 1. Effect of ethanol (0.5, 1.0 and 2.0 g/kg) on the exploratory activity of male and female CCK_2 receptor deficient mice in the elevated plus-maze ($N = 8$–10 per group). $^{*}p < 0.05$, $^{**}p < 0.01$: Tukey's HSD test, ethanol compared to saline treatment of respective genotype. White bars: saline; striped bars: ethanol 0.5 g/kg; hatched bars: ethanol 1.0 g/kg; black bars: ethanol 2.0 g/kg.

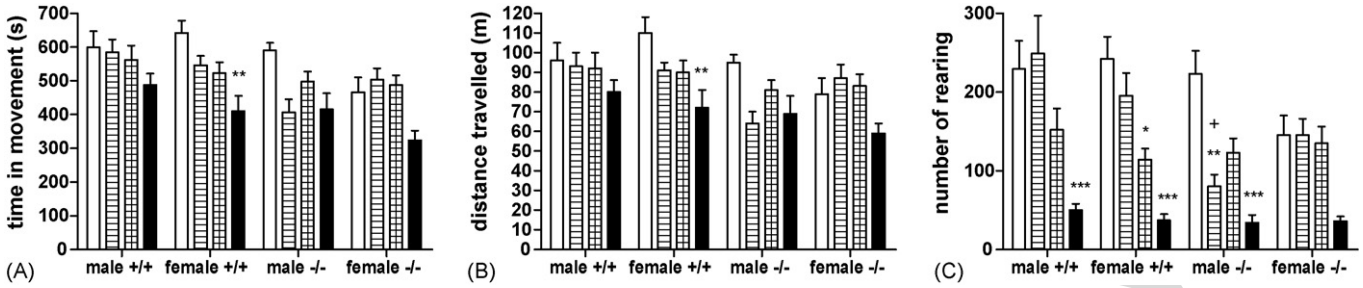

Fig. 2. Effect of ethanol (0.5, 1.0 and 2.0 g/kg) on the locomotor activity of male and female CCK_2 receptor deficient mice ($N = 10$–12 per group). $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$: Tukey's HSD test, ethanol compared to saline treatment of respective genotype. $^{+}p < 0.001$: statistically significant difference between male wild-type (+/+) and male CCK_2 receptor deficient mice (−/−) of ethanol dose 0.5 g/kg. White bars: saline; striped bars: ethanol 0.5 g/kg; hatched bars: ethanol 1.0 g/kg; black bars: ethanol 2.0 g/kg.

3.2. Locomotor activity test

(1) *Time in movement*. Three-way independent-groups ANOVA (genotype × gender × ethanol treatment) established significant effects of genotype ($F(1, 167) = 20.46$, $p < 0.001$), ethanol treatment ($F(3, 167) = 13.85$, $p < 0.001$) and genotype × gender × ethanol treatment interaction ($F(3, 167) = 2.82$, $p < 0.05$). At the highest dose (2.0 g/kg), ethanol induced a significant suppression of locomotor activity only in female wild-type mice ($p < 0.01$) (Fig. 2A). It should be noted, though, that the baseline activity of female mutant mice (−/−) tended to be lower compared to their wild-type littermates (+/+) ($p = 0.07$). In male mice, lacking CCK_2 receptors (−/−), but not in their wild-type littermates (+/+), ethanol tended to suppress time in movement at doses 0.5 g/kg ($p = 0.08$) and 2.0 g/kg ($p = 0.07$) (Fig. 2A).

(2) *Distance travelled*. Significant effects of genotype ($F(1, 167) = 15.50$, $p < 0.001$), ethanol treatment ($F(3, 167) = 9.49$, $p < 0.001$) and genotype × gender × ethanol treatment interaction ($F(3, 167) = 2.94$, $p < 0.05$) were demonstrated. Significant decrease in locomotor activity after administration of the highest dose of ethanol (2.0 g/kg) was observed only in female wild-type mice (+/+) ($p < 0.01$) (Fig. 2B). Again, the baseline activity of female mutant mice (−/−) tended to be lower compared to their wild-type littermates (+/+) ($p = 0.09$). In male mice, ethanol administration did not significantly affect the distance travelled.

(3) *Number of rearing*. Three-way ANOVA indicated significant effects of genotype ($F(1, 167) = 12.76$, $p < 0.001$), ethanol treatment ($F(3, 167) = 35.40$, $p < 0.001$), genotype × ethanol treatment interaction ($F(3, 167) = 4.11$, $p < 0.01$) and genotype × gender × ethanol treatment interaction ($F(3, 167) = 3.22$, $p < 0.05$). Ethanol significantly suppressed rearing at the highest dose used (2.0 g/kg) in all groups, except for female mutant (−/−) mice. The same effect was induced in female wild-type mice (+/+) by dose 1.0 g/kg ($p < 0.05$) and in the male mutant mice (−/−) by dose 0.5 g/kg ($p < 0.01$) (Fig. 2C). The suppression of rearing behaviour induced by the small dose of ethanol (0.5 g/kg) in male mice lacking CCK_2 receptors (−/−) was significant when compared to the respective treatment in their wild-type littermates (+/+) ($p < 0.001$) (Fig. 2C).

Table 1
The time to loss of righting reflex (LORR, s), the time to regain of righting reflex (RRR, min) and the blood ethanol concentrations at the regain of the righting reflex (BEC_{RRR}, g/l) in wild-type mice (+/+) and in mice, lacking CCK_2 receptors (−/−) after administration of ethanol 4.0 g/kg

Group	LORR (s)	RRR (min)	BEC_{RRR} (g/l)
Male (+/+)	74 ± 2.8	56 ± 7.9	3.36 ± 0.08
Female (+/+)	75 ± 2.0	40 ± 4.4	3.57 ± 0.07
Male (−/−)	75 ± 2.0	50 ± 7.4	3.19 ± 0.05
Female (−/−)	79 ± 3.0	51 ± 7.9	3.12 ± 0.07***

Number of mice, $N = 14$–15 per group.
*** $p < 0.001$: Tukey's HSD test, significant difference between female wild-type (+/+) and female mutant (−/−) mice.

3.3. Loss of righting reflex

(1) *Time to loss of righting reflex*. Two-way independent-groups ANOVA (genotype × gender) revealed no significant effects in the time to loss of righting reflex.

(2) *Time to regain of righting reflex*. No genotype- or gender-related differences were observed in the time to regain of righting reflex.

(3) *Blood ethanol concentration at regain of righting reflex*. Significant effects of genotype ($F(1, 54) = 22.82$, $p < 0.001$) and genotype × gender interaction ($F(1, 54) = 4.47$, $p < 0.05$) were demonstrated by two-way ANOVA. Blood ethanol concentrations at regain of righting reflex were significantly lower in female mice lacking CCK_2 receptors (−/−), than in their wild-type (+/+) littermates ($p < 0.001$). No genotype-related difference was established in male mice. The data are summarised in Table 1.

3.4. Blood ethanol concentrations (BECs)

Four-way mixed-design ANOVA (genotype × gender × dose × time) established significant effects of ethanol dose ($F(1, 37) = 671.53$, $p < 0.001$), time ($F(3, 111) = 943.84$, $p < 0.001$), genotype × time interaction ($F(3, 111) = 4.18$, $p < 0.01$), gender × time interaction ($F(3, 111) = 5.33$, $p < 0.01$), dose × time interaction ($F(3, 111) = 40.29$, $p < 0.001$), genotype × gender × time interaction ($F(3, 111) = 3.48$, $p < 0.05$) and genotype × dose × time interaction ($F(3, 111) = 3.35$, $p < 0.05$).

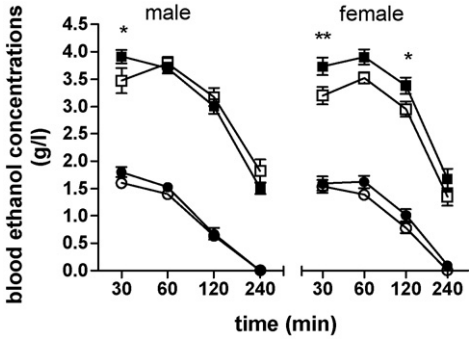

Fig. 3. Blood ethanol concentrations (g/l) in male and female mice, lacking CCK_2 receptors (−/−), after injection of ethanol 2.0 or 4.0 g/kg ($N = 6$ per group). $^{*}p < 0.05$, $^{**}p < 0.01$: Tukey's HSD test, wild-type mice (+/+) compared to CCK_2 receptor deficient mice (−/−) at respective time point after injection of ethanol 4.0 g/kg. Filled squares: wild-type mice (+/+), ethanol dose 4.0 g/kg; open squares: mutant mice (−/−), ethanol dose 4.0 g/kg; filled circles: wild-type mice (+/+), ethanol dose 2.0 g/kg; open circles: mutant mice (−/−), ethanol dose 2.0 g/kg.

Blood ethanol concentrations were significantly lower in female mice, lacking CCK_2 receptors (−/−), when compared to female wild-type mice (+/+), 30 and 120 min after the injection of ethanol at a dose 4.0 g/kg ($p < 0.01$ and $p < 0.05$, respectively) (Fig. 3). In male mutant mice (−/−) BECs were also significantly lower than in their wild-type littermates (+/+) 30 min after injection of ethanol 4.0 g/kg ($p < 0.05$) (Fig. 3). There were no genotype- or gender-related differences after injection of ethanol 2.0 g/kg.

3.5. Ethanol preference and intake

(1) *Ethanol preference*. Three-way mixed-design ANOVA (genotype × gender × ethanol concentration) indicated significant effects of gender ($F(1, 56) = 15.34$, $p < 0.001$), ethanol concentration ($F(2, 112) = 30.03$, $p < 0.001$) and gender × ethanol concentration interaction ($F(2, 112) = 9.39$, $p < 0.001$). Ethanol preference at concentrations 6% and 10% was significantly higher in both female wild-type mice (+/+) ($p < 0.05$ and $p < 0.001$, respectively) and female mutant mice (−/−) ($p < 0.01$ and $p < 0.001$, respectively) when compared to their male littermates (Fig. 4A).

(2) *Ethanol intake*. Significant effects of genotype ($F(1, 56) = 6.55$, $p < 0.05$), gender ($F(1, 56) = 44.88$, $p < 0.001$), ethanol concentration ($F(2, 112) = 212.28$, $p < 0.001$), genotype × ethanol concentration interaction ($F(2, 112) = 5.12$, $p < 0.01$) and gender × ethanol concentration interaction ($F(2, 112) = 34.34$, $p < 0.001$) were established. *Post hoc* comparison between groups demonstrated significant gender- and genotype-related differences in ethanol consumption at concentration 10%. Female mice, independent of genotype, consumed significantly more ethanol than their male littermates (6% ethanol: $p < 0.05$ for wild-type (+/+) mice and $p < 0.01$ for mutant (−/−) mice; 10% ethanol: $p < 0.001$ for both wild-type (+/+) and mutant (−/−) mice) (Fig. 4B). Moreover, female mice, lacking CCK_2 receptors (−/−), consumed significantly more 10% ethanol solution than their wild-type littermates (+/+) ($p < 0.001$) (Fig. 4B).

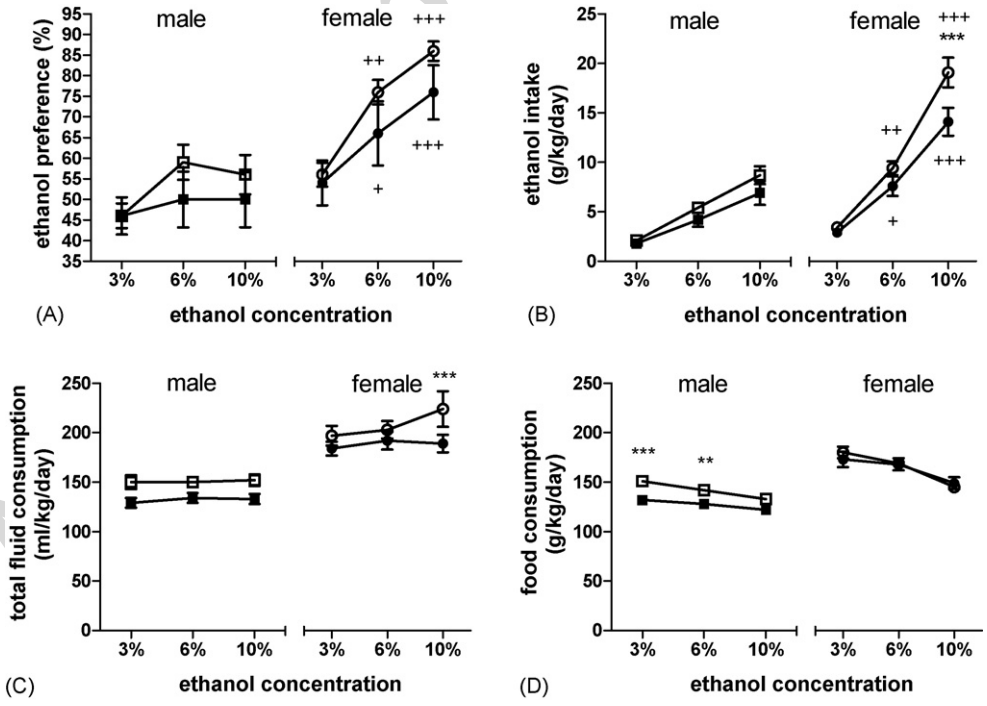

Fig. 4. Ethanol preference and intake, and total liquid and food intake in CCK_2 receptor deficient mice during voluntary ethanol consumption ($N = 15$ per group). $^{**}p < 0.01$, $^{***}p < 0.001$: Tukey's HSD test, wild-type mice (+/+) compared to mice, lacking CCK_2 receptors (−/−) at respective ethanol concentration. $^{+}p < 0.05$, $^{++}p < 0.01$, $^{+++}p < 0.001$: female mice compared to male mice of respective genotype at respective ethanol concentration. Filled squares: male wild-type (+/+) mice; open squares: male mutant mice (−/−); filled circles: female wild-type (+/+) mice; open circles: female mutant mice (−/−).

Table 2
Sucrose and quinine preference (%) in mice, lacking CCK_2 receptors (−/−)

Group	Sucrose, 1.7%	Sucrose, 4.3%	Quinine, 0.03 mM	Quinine, 0.10 mM
Male (+/+)	89 ± 1.6	97 ± 0.6	55 ± 4.7	43 ± 5.6
Female (+/+)	89 ± 1.5	97 ± 0.5	55 ± 5.0	39 ± 5.2
Male (−/−)	90 ± 1.1	97 ± 0.3	56 ± 4.1	42 ± 4.0
Female (−/−)	90 ± 1.4	96 ± 0.5	61 ± 4.4	49 ± 5.8

Number of mice $N = 15$ per group. No significant differences were observed in taste preference, compared to wild-type mice (+/+).

3.6. Total fluid and food consumption, and body weight changes during alcohol intake test

(1) *Total fluid consumption*. Significant effects of genotype ($F(1, 56) = 6.64$, $p < 0.05$), gender ($F(1, 56) = 56.47$, $p < 0.001$) and ethanol concentration ($F(2, 112) = 3.16$, $p < 0.05$) were established by three-way mixed-design ANOVA (genotype × gender × ethanol concentration). Total liquid intake per body weight at ethanol concentration 10% was significantly higher ($p < 0.001$) in female mice, lacking CCK_2 receptors (−/−) compared to their wild-type littermates (+/+) (Fig. 4C). Genotype-related difference in the total fluid intake was not observed in male mice.

(2) *Food consumption*. Significant effects of gender ($F(1, 56) = 52.28$, $p < 0.001$), ethanol concentration ($F(2, 112) = 68.56$, $p < 0.001$), genotype × ethanol concentration interaction ($F(2, 112) = 3.46$, $p < 0.05$) and gender × ethanol concentration interaction ($F(2, 112) = 10.29$, $p < 0.001$) were demonstrated. Male mice, lacking CCK_2 receptors (−/−), consumed significantly more food per body weight than their wild-type (+/+) littermates at ethanol concentrations 3% ($p < 0.001$) and 6% ($p < 0.01$) (Fig. 4D). No such difference was observed in female mice.

(3) *Body weight changes*. Three-way ANOVA indicated significant effects of gender ($F(1, 56) = 158.11$, $p < 0.001$) and gender × ethanol concentration interaction ($F(2, 112) = 16.29$, $p < 0.001$). While there were significant differences between male and female mice, no genotype-related differences were observed (data not shown).

3.7. Taste preference

(1) *Sucrose preference*. Three-way mixed-design ANOVA (genotype × gender × sucrose concentration) revealed significant effect of sucrose concentration ($F(1, 56) = 132.85$, $p < 0.001$). *Post hoc* comparison did not reveal any differences between the groups.

(2) *Quinine preference*. Significant effect of quinine concentration ($F(1, 56) = 47.77$, $p < 0.001$) was demonstrated. No significant genotype- or gender-related differences were observed. The data on taste preference are summarised in Table 2.

4. Discussion

In the present study we demonstrate that the behavioural effects of ethanol are altered in mice, lacking CCK_2 receptors (−/−), and that these alterations are gender-specific. Ethanol induced a significant reduction in anxiety-related behaviours (increase of time spent on open arms, the number of unprotected head-dips and lines crossed) in the plus-maze in female mice, lacking CCK_2 receptors (−/−). Moreover, this effect was observed at two doses (1.0 and 2.0 g/kg), indicating shift in sensitivity to anxiolytic properties of ethanol in female mutant mice. This finding was coupled with the increased sensitivity to sedative effect of ethanol (4.0 g/kg) in female mutant mice (−/−). Although the duration of the loss of righting reflex was not significantly altered, female homozygous (−/−) mice had significantly lower blood ethanol concentrations at regain of the righting reflex (BEC_{RRR}) compared to their wild-type littermates (+/+). Given the fact that alcohol exerts many of its effects in the CNS through GABA-ergic system [10,14] our current results are in accordance with the evidence in favour of altered activity of GABA-ergic system in female CCK_2 receptor deficient mice (−/−) [33,34]. According to the study of Raud et al. [34], female mice lacking CCK_2 receptors, have an increased tone of GABA-ergic system, a reduced basal anxiety and an increased sensitivity to diazepam-induced impairments in motor coordination. Moreover, Raud et al. [33] demonstrated 1.6-fold increase in expression of the α2 subunit of $GABA_A$ receptors in the frontal cortex of CCK_2 receptor deficient mice. It has been shown that this subunit mediates the anxiolytic action of diazepam and that the genetic invalidation of this gene abolishes this effect of diazepam [24,28]. While certain genotype-related differences in the anxiolytic and sedative effects of ethanol were established in the current study in female mice, none of these were observed in male mice.

While there was an increase in sensitivity to anxiolytic and sedative properties of ethanol in female mutant mice (−/−), ethanol-induced locomotor suppression was modified in female wild-type (+/+) mice. Ethanol (2.0 g/kg) induced a significant reduction of both horizontal (distance travelled) and vertical (number of rearing) activity in female wild-type (+/+) mice. However, one should take into account that these effects of ethanol are probably apparent due to the higher basal locomotor activity in wild-type (+/+) mice compared to their homozygous (−/−) littermates. In male mice, ethanol induced a significant genotype-dependent suppression of vertical (number of rearing), but not horizontal (distance travelled) activity. While in male wild-type (+/+) mice ethanol decreased the frequency of rearing only at the largest dose used (2.0 g/kg), significant suppression of rearing in male mutant (−/−) mice was observed at doses 0.5 and 2.0 g/kg. Acute administration of ethanol has been reported

to increase striatal dopamine at lower doses, and decrease it at higher doses [3,4]. According to our previous reports, male, but not female mice, lacking CCK_2 receptors (−/−), display altered properties of striatal dopamine neurotransmission and altered effects of dopaminergic drugs on locomotor activity [1,19]. We have previously hypothesised that in male mice, lacking CCK_2 receptors (−/−), there is an increase in sensitivity of presynaptic dopamine D_2 receptors that might account for the reduction of locomotor activity after administration of small doses of apomorphine or amphetamine. Recently, Rünkorg et al. [37] demonstrated the reduced expression of dopamine D_2 receptors in the mesencephalon of male CCK_2 receptor deficient mice. Dopamine D_2 receptors in the mesencephalon are dopamine autoreceptors. These receptors regulate the release of dopamine and it has been suggested that autoreceptor subsensitivity contributes to the enhanced release of dopamine [31]. Moreover, it is interesting to note that Miyasaka et al. [27] revealed a reduced level of dopamine D_2 receptor protein in the nucleus accumbens of homozygous (−/−) CCK_2 receptor deficient mice compared to their wild-type (+/+) littermates. Further studies are needed to establish a link between ethanol-induced suppression of activity and changes in the dopaminergic system of male mice, lacking CCK_2 receptors (−/−). It is to be emphasized here, that in the present study modified effect of ethanol on locomotor activity is the only alteration demonstrated in male mice due to invalidation of CCK_2 receptors.

Blood ethanol concentrations (BECs) after injection of ethanol 2.0 g/kg were not significantly affected by either genotype or gender, implicating that the behavioural results reported here are not due to altered ethanol metabolism. However, significantly lower blood ethanol concentrations were observed after injection of ethanol 4.0 g/kg in mice, lacking CCK_2 receptors (−/−). While in male mutant (−/−) mice BECs were significantly lower only 30 min after ethanol injection, in female mice significant differences were found 30 and 120 min after ethanol administration. Altered blood ethanol kinetics after dose 4.0 g/kg seems to have confounded the loss of righting reflex test. While the duration of the loss of righting reflex was not affected by genotype or gender, the blood ethanol concentrations at regain of righting reflex were significantly lower in female CCK_2 receptor deficient mice (−/−). This finding likely reflects the higher sensitivity of female homozygous (−/−) mice to the sedative action of ethanol, but this effect cannot be demonstrated by measuring of loss of righting reflex because of accelerated ethanol kinetics in female mutant mice (−/−).

In the current study we report a gender-specific alteration of alcohol intake in CCK_2 receptor deficient mice. Female, but not male mice, lacking CCK_2 receptors (−/−), consumed significantly more 10% ethanol than their wild-type littermates (+/+). This finding is in good accordance with the study of Miyasaka et al. [27] reporting unaltered ethanol consumption in CCK_2 receptor deficient (−/−) male mice. However, higher ethanol intake in female mutant mice (−/−) was accompanied by significant increase in total fluid intake at alcohol concentration 10%, resulting in unaltered ethanol preference. Provided that female mice, lacking CCK_2 receptors (−/−), have increased sensitivity to anxiolytic and sedative effects of ethanol, increased ethanol intake is somewhat unexpected finding. To exclude alterations in the taste preference, that could have influenced ethanol intake, preference to non-alcoholic tastants sucrose and quinine was measured in mice, lacking CCK_2 receptors (−/−). However, no genotype or gender-related alterations were observed in taste preference. Further studies are suggested to determine if the development of tolerance to ethanol is affected by the invalidation of CCK_2 receptors in female mice. It has to be mentioned, that the background strain of homozygous (−/−) and wild-type (+/+) mice in the current study was 129Sv/C57BL/6 back-crossed six times to C57BL/6. C57BL/6 is known for its high spontaneous ethanol preference and consumption [2,26]. Thus, the phenotype reported here might be specific to current background strain and could be modified in strains with lower ethanol intake.

Female mice, independent of genotype, preferred and consumed ethanol significantly more than their male counterparts, especially at higher concentrations (6% and 10%). This is in line with other studies reporting increased ethanol preference in female compared to male rodents [21,25,26], making studies in both genders preferable to the common practice of using only male rodents. It is to be stressed, based on our previous report [1] and the present results, that employing both genders seems to be especially relevant in studying CCK_2 receptor deficient mice, as some of alterations caused by this mutation are observed in male, while the others in female mice.

In conclusion, the present study demonstrates that both male and female mice, lacking CCK_2 receptors, display altered behavioural responses to the administration of ethanol. Ethanol-induced anxiolytic and sedative, but not locomotor suppressing, responses were enhanced in female mice lacking CCK_2 receptors. Conversely, in male mutant mice only altered locomotor effect of ethanol was established. While ethanol consumption was significantly increased in female mice, lacking CCK_2 receptors (−/−), no genotype-related difference was observed in male mice. Taken together, these data confirm gender-specific phenotype, induced by the invalidation of CCK_2 receptor gene, and favour studies employing both male and female transgenic mice. The behavioural effects of ethanol in male homozygous (−/−) mice might partly result from an increased function of dopaminergic system, established in previous studies [12,19]. By contrast, the alterations in the action of ethanol in female genetically modified animals could be attributed to the elevated activity of GABA-ergic system shown previously [33,34]. Further research is encouraged to elucidate mechanisms of gender-specific increase in ethanol intake and its relation to altered behavioural effects of ethanol in mice, lacking CCK_2 receptors.

Acknowledgments

This study was financially supported by the grants from the Estonian Science Foundation (GARFS 6590 and GARFS 5688), the Estonian Ministry of Education and Science (0182584Bs03) and VARMC-TIPP.

References

[1] Abramov U, Raud S, Kõks S, Innos J, Kurrikoff K, Matsui T, et al. Targeted mutation of CCK(2) receptor gene antagonises behavioural changes induced by social isolation in female, but not in male mice. Behav Brain Res 2004;155:1–11.

[2] Belknap JK, Crabbe JC, Young ER. Voluntary consumption of ethanol in 15 inbred mouse strains. Psychopharmacology 1993;112:503–10.

[3] Blanchard BA, Steindorf S, Wang S, Glick SD. Sex differences in ethanol-induced dopamine release in nucleus accumbens and in ethanol consumption in rats. Alcohol Clin Exp Res 1993;17:968–73.

[4] Budygin EA, Phillips PE, Robinson DL, Kennedy AP, Gainetdinov RR, Wightman RM. Effect of acute ethanol on striatal dopamine neurotransmission in ambulatory rats. J Pharmacol Exp Ther 2001;297:27–34.

[5] Costall B, Domeney AM, Hughes J, Kelly ME, Naylor RJ, Woodruff GN. Anxiolytic effects of CCK-B antagonists. Neuropeptides 1991;19:65–73.

[6] Crawley JN. Interactions between cholecystokinin and other neurotransmitter systems. In: Bradwejn J, Vasar E, editors. Cholecystokinin and anxiety: from neuron to behaviour. Austin: Springer-Verlag/R.G. Landes Company; 1995. p. 101–26.

[7] Crawley JN, Corwin RL. Biological actions of cholecystokinin. Peptides 1994;15:731–55.

[8] Crespi F. The role of cholecystokinin (CCK), CCK-A or CCK-B receptor antagonists in the spontaneous preference for drugs of abuse (alcohol or cocaine) in naive rats. Meth Find Exp Clin Pharmacol 1998;20:679–97.

[9] Crespi F, Corsi M, England T, Ratti E, Trist DG, Gaviraghi G. Spontaneous preference for ethanol in naive rats is influenced by cholecystokinin A receptor antagonism. Alcohol 1997;14:327–32.

[10] Criswell HE, Breese GR. A conceptualisation of integrated actions of ethanol contributing to its GABAmimetic profile: a commentary. Neuropsychopharmacology 2005;30:1407–25.

[11] Croft AP, Brooks SP, Cole J, Little HJ. Social defeat increases alcohol preference of C57BL/10 strain mice; effect prevented by a CCKB antagonist. Psychopharmacology (Berl) 2005;183:163–70.

[12] Daugé V, Beslot F, Matsui T, Roques BP. Mutant mice lacking the cholecystokinin2 receptor show a dopamine-dependent hyperactivity and a behavioural sensitization to morphine. Neurosci Lett 2001;306:41–4.

[13] Daugé V, Lena I. CCK in anxiety and cognitive processes. Neurosci Biobehav Rev 1998;22:815–25.

[14] Davies M. The role of GABAA receptors in mediating the effects of alcohol in the central nervous system. J Psychiat Neurosci 2003;28:263–74.

[15] DiBattista D, McKenzie TLB, Hollis-Walker L. Cholecystokinin reduces ethanol consumption in golden hamsters. Alcohol 2003;29:173–81.

[16] Geary N, Wolfe A, Polidori C, Policani F, Massi M. Exogeneous and endogenous CCK inhibit ethanol ingestion in Sardinian alcohol-preferring rats. Peptides 2004;25:1185–94.

[17] Handley SL, Mithani S. Effects of alpha-adrenoceptor agonists and antagonists in a maze-exploration model of 'fear'-motivated behaviour. Naunyn-Schmiedebergs Arch Pharmacol 1984;327:1–5.

[18] Harro J, Wahlstrom G, Oreland L. Cholecystokinin receptor binding after long-term ethanol treatment in rats. Alcohol Alcohol 1994;29:575–81.

[19] Kõks S, Volke V, Veraksits A, Rünkorg K, Sillat T, Abramov U, et al. Cholecystokinin2 receptor-deficient mice display altered function of brain dopaminergic system. Psychopharmacology (Berl) 2001;58:198–204.

[20] Kulkosky PJ. Neuropeptidergic control of ethanol intake. J Toxicol Toxin Rev 1996;15:341–51.

[21] Lancaster FE, Spiegel KS. Sex differences in patterns of drinking. Alcohol 1992;9:415–20.

[22] Lister RG. The use of a plus-maze to measure anxiety in the mouse. Psychopharmacology (Berl) 1987;92:180–5.

[23] Little HJ, O'Callaghan MJ, Butterworth AR, Wilson J, Cole J, Watson WP. Low alcohol preference among the "high alcohol preference" C57 strain of mice; preference increased by saline injections. Psychopharmacology (Berl) 1999;147:182–9.

[24] Löw K, Crestani F, Keist R, Benke D, Brünig I, Benson JA, et al. Molecular and neuronal substrate for the selective attenuation of anxiety. Science 2000;290:131–4.

[25] Meliska CJ, Bartke A, McGlacken G, Jensen RA. Ethanol, nicotine, amphetamine, and aspartame consumption and preferences in C57BL/6 and DBA/2 mice. Pharmacol Biochem Behav 1995;50:619–26.

[26] Middaugh LD, Kelley BM, Bandy AL, McGroarty KK. Ethanol consumption by C57BL/6 mice: influence of gender and procedural variables. Alcohol 1999;17:175–83.

[27] Miyasaka K, Hosoya H, Takano S, Ohta M, Sekime A, Kanai S, et al. Differences in ethanol ingestion between cholecystokinin-A receptor deficient and -B receptor deficient mice. Alcohol Alcohol 2005;40:176–80.

[28] Möhler H, Fritschy JM, Rudolph U. A new benzodiazepine pharmacology. Pharmacol Exp Ther 2002;300:2–8.

[29] Nagata A, Ito M, Iwata N, Kuno J, Takano H, Minowa O, et al. G protein-coupled cholecystokinin-B/gastrin receptors are responsible for physiological cell growth of the stomach mucosa in vivo. Proc Natl Acad Sci USA 1996;93:11825–30.

[30] Noble F, Wank SA, Crawley JN, Bradwejn J, Seroogy KB, Hamon M, et al. International Union of Pharmacology. XXI. Structure, distribution, and functions of cholecystokinin receptors. Pharmacol Rev 1999;51:745–81.

[31] Pierce RC, Kalivas PW. A circuitry model of the expression of behavioral sensitization to amphetamine-like psychostimulants. Brain Res Rev 1997;25:192–216.

[32] Pommier B, Beslot F, Simon A, Pophillat M, Matsui T, Daugé V, et al. Deletion of CCK_2 receptor in mice results in an upregulation of the endogenous opioid system. J Neurosci 2002;22:2005–11.

[33] Raud S, Innos J, Abramov U, Reimets A, Kõks S, Soosaar A, et al. Targeted invalidation of CCK(2) receptor gene induces anxiolytic-like action in light–dark exploration, but not in fear conditioning test. Psychopharmacology (Berl) 2005;181:347–57.

[34] Raud S, Rünkorg K, Veraksits A, Reimets A, Nelovkov A, Abramov U, et al. Targeted mutation of CCK_2 receptor gene modifies the behavioural effects of diazepam in female mice. Psychopharmacology (Berl) 2003;168:417–25.

[35] Rehfeld JF, Nielsen FC. Molecular forms and regional distribution of cholecystokinin in the central nervous system. In: Bradwejn J, Vasar E, editors. Cholecystokinin and anxiety: from neuron to behaviour. Austin: Springer-Verlag/R.G. Landes Company; 1995. p. 33–56.

[36] Rotzinger S, Vaccarino FJ. Cholecystokinin receptor subtypes: role in the modulation of anxiety-related and reward-related behaviours in animal models. J Psychiat Neurosci 2003;28:171–81.

[37] Rünkorg K, Värv S, Matsui T, Kõks S, Vasar E. Differences in behavioural effects of amphetamine and dopamine-related gene expression in wild-type and homozygous CCK_2 receptor deficient mice. Neurosci Lett 2006;406:17–22.

[38] Rünkorg K, Veraksits A, Kurrikoff K, Luuk H, Raud S, Abramov U, et al. Distinct changes in the behavioural effects of morphine and naloxone in CCK_2 receptor-deficient mice. Behav Brain Res 2003;144:125–35.

[39] Toth P, Shaw C, Perlanski E, Grupp LA. Cholecystokinin octapeptide reduces ethanol intake in food- and water-sated rats. Pharmacol Biochem Behav 1990;35:493–5.

[40] Veraksits A, Rünkorg K, Kurrikoff K, Raud S, Abramov U, Matsui T, et al. Altered pain sensitivity and morphine-induced anti-nociception in mice lacking CCK_2 receptors. Psychopharmacology (Berl) 2003;166:168–75.

[41] Weatherford SC, Figlewicz DP, Park CR, Woods SC. Chronic alcohol consumption increases sensitivity to the anorexic effect of cholecystokinin. Am J Physiol 1993;265:211–5.

[42] Wilson J, Little HJ. CCK(B) antagonists protect against some aspects of the ethanol withdrawal syndrome. Pharmacol Biochem Behav 1998;59:967–73.

[43] Wilson J, Watson WP, Little HJ. CCK(B) antagonists protect against anxiety-related behaviour produced by ethanol withdrawal, measured using the elevated plus-maze. Psychopharmacology (Berl) 1998;137:120–31.